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14. ABSTRACT

Over the past four years, we have optimized various systems for the identification of tumor-reactive T cells, and generated tumor-reactive T cell clones from a number of breast cancer patient samples. We have worked out killing assays to screen breast cancer patients' tumor specific CD8+ T cells against autologous tumor cells and breast cancer cell lines. We have identified TCR sequences from patients BC166 and BC81 CD8 T cell clones and downstream analysis of antigen specificity will be assessed by the Denver team. The identification of these T cells will lead to downstream antigen discovery.

15. SUBJECT TERMS

Breast cancer, immunotherapy, vaccine, antigens

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Final Progress Report
DoD Multi-Team Award
Enhancing the Breadth and Efficacy of Therapeutic Vaccines for Breast Cancer
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INTRODUCTION

The immune response offers exquisite specificity and the potential to target tumor cells without harming normal cells. Inducing an effective immune response via therapeutic vaccines for cancer had been a promising but elusive goal for years. For breast cancer (BC), vaccine efforts have largely focused on eliciting immune responses to HER2. While HER2 is generally assumed to be a good antigen in HER2-overexpressing tumors, HER2-specific T cells exist at very low levels (less than 0.1%) in peripheral blood of such patients [1]. Hierarchy of the T cell repertoire and negative selection can shape immune responses in ways not readily predictable from protein expression levels alone. Thus, targeting a single antigen such as HER2 in breast cancer is likely to be insufficient - instead we need a repertoire of multiple immunologically validated T cell antigens present in breast cancers that can be deployed in a patient-specific manner. Research has focused on stimulating T cells using many pathways including the T cell antigen receptor (TCR), via co-stimulatory pathways, and manipulating the tumor environment. To optimally activate pre-existing anti-tumor T cells in BC patients, the antigens to which these T cells target must be determined. It is now recognized that invasive ductal carcinoma of the breast is a heterogeneous disease consisting of several major molecularly defined subtypes, including Luminal A, Luminal B, HER2+, and Basal (also known as 'triple-negative', and includes the 'claudin-low' subset). These subtypes have distinct clinical, genomic and proteomic features, and it is becoming clear that there are differences between BC subtype and response to specific therapeutic agent. These results, combined with the differences in gene expression that define the distinct subtypes, make it likely that each BC subtype elicits immune responses via distinct sets of antigens, and may evade T cell-mediated killing by distinct mechanisms. Based on these newly discovered features of BC and the host immune response, this project seeks to develop a robust portfolio of immunologically validated antigens for the major BC subtypes, including those that target breast cancer stem cells, that can be used in a patient-specific manner for therapeutic vaccination, as well as to identify drugs that can synergize with these novel immunotherapies. The ultimate goal is to match these antigens and drugs to each patient's tumor subtype, thereby treating each patient with the most potent combinations and opening the door to personalized immunotherapy for breast cancer. This multi-ream project will use a number of novel immunological approaches to look for evidence of BC subtype specific tumor-reactive T cells within the tumor and/or tumor-draining lymph nodes (TDLNs) including isolating, expanding and cloning tumor-reactive T cells which will culminate in a robust portfolio of immunologically validated antigens for the major breast cancer subtypes, including those that target breast cancer stem cells. We seek to expand and enhance the function of these pre-existing anti-tumor T cells in patients by discovering their natural antigens, and identifying mimotopes that broadly activate them with even higher potency. Furthermore, we will enhance the efficacy of these T cells by identifying existing drugs that promote cancer cell apoptosis but have little or no negative effect on T cells. All of these antigens and agents can be matched to each patient's tumor subtype and other molecular characteristics, thereby opening the door to personalized immunotherapy.

BODY:

Our team at City of Hope consisted of 1 assistant research professor, 1 post-doctoral fellow, and 3 research associates. We have worked closely with our surgery, medical oncology, and pathology colleagues via an honest broker to obtain samples from the operating room to pathology and to my laboratory. In addition, we continually refined our protocols to maximize recovery of immune cells from tumor and lymph node specimens, and to optimize methods for analysis of fresh samples by flow cytometry. Below is a summary of our progress over the four years of our award in relation to our proposed SOW tasks:

Identify immunologically validated antigens by determining antigens recognized by anti-tumor T cells from patients with major subtypes of breast cancer.

1. Generate reagents and identify conditions for experiments to follow: months 1-40, Lee, Slansky, and Spellman
2. Enroll 100 patients with all major breast cancer subtypes from the City of Hope Cancer Center (CoH): months 1-36, Lee
3. Process patient samples (blood, TDLNs, tumor): months 1-38, Lee
4. Identify and isolate anti-tumor T cells from TDLNs and tumor samples: months 1-40, Lee
5. Generation and initial analysis of T cell clones: months 1-40, Lee
6. Determine antigens as subtype-specific, stem-specific, or shared (Aim 4a): months 12- 40, Lee, Slansky and Spellman
7. Identify antigens that target breast cancer stem cells (Aim 3b): months 12-40, Lee, Slansky and Spellman

Patient Enrollment and Sample Acquisition

Our progress for the past four years has largely focused on tasks 1-5. In 2016, we have acquired specimens from 25 additional breast cancer patients consisting of peripheral blood, lymph node, and/or tumor. Clinical characteristics are summarized in Table 1. At the end of year four, we have enrolled a total of 69 breast cancer patients for these studies. From those 69 patients, we have sent out a total of 120 samples to the Slansky group for TCR sequencing. All participants were without a history of any immune disorder prior to breast cancer diagnosis and had their surgical treatments at City of Hope (CoH). Through an honest broker, written informed consent had been obtained from all participants according to CoH, DoD HSRRB, and HIPAA regulations using a tissue banking protocol. Patient peripheral blood samples, breast tumor tissue, and/or tumor draining lymph node (TDLN: non-sentinel lymph node and/or sentinel lymph node) were collected and have been utilized for research purposes.

As mentioned in our previous reports, our approach to identifying tumor-reactive T cells has focused solely on CD8+ T cells primarily due to the fact that most of our protocols and tools (antibodies, identified peptides, and peptide libraries) are restricted to HLA-A2, which is only useful for CD8+ T cells. Our original plan to focus on HLA-DR4 restricted CD4s has proven difficult since there is no publically available antibody for HLA-DR4 and since very few of our HLA-A2+ patients have also been HLA-DR4+ (Table 2).

<u>Human leukocyte antigen (HLA) typing</u>

We have arranged to have patient human leukocyte antigen (HLA) genes genotyped through the Histocompatibility lab here at CoH. CoH's Histocompatibility Laboratory is fully accredited by The American Society of Histocompatibility and Immunogenetics (ASHI), College of American Pathologists (CAP), and Clinical Laboratory Improvement Amendments (CLIA 88). They will carry out the typing using the sequence-specific oligonucleotide probe (SSOP) method. The SSOP method allows the HLA lab to define the HLA type of our patient subjects to the allele level (so called '4 digits'). We have requested allele level typing of subjects for only HLA-A2 and HLA-DR for 33 patients overall, but information on other alleles is available at a later date if desired. As such, we have fully HLA typed 4 breast cancer patients in which we have identified tumor-reactive T cells (Table 3).

Identify and isolate anti-tumor T cells from TDLNs and tumor samples

Growth of Autologous Cancer Cells

Having a source of autologous cancer cells are an ideal target source for identification of bonafide tumor-reactive T cells. In our previous report, we described how we cultured and grew autologous cancer cells in vitro. Reactivity assays done with BC166 reactive T cells showed a loss of tumor-reactivity when reacted against primary tumor cells cultured in vitro for greater than two weeks (data not shown). As a result, we heavily favor reacting patient T cells against either short-term (less than five days) or cryopreserved tumor cells.

Generation and analysis of T cell clones

Identification and characterization of tumor T cells ex vivo

As described in previous reports, we have optimized conditions for the identification of tumor reactive T cells. The two previous primary strategies were to use mDCs pulsed with breast cancer cell line lysate and to use autologous tumor cells as targets. Additionally, we have examined CD137 expression ex vivo to identify naturally occurring tumor reactive T cells as a sign of in situ activation in the tumor. Additionally, we further characterized the T cells from breast cancer patients ex vivo to determine their phenotypic and activation status. The clinical characteristics of these patients are shown in Table 4. Briefly, after mechanically disassociating and enzymatically digesting the tumor and/or tumor positive TDLN into single cell suspensions, the cells were stained with CD3, CD8, CD4, CD45RA, and CCR7 for markers of T cell differentiation, and CD8, CD137, OX-40, CD25, HLA-DR, Ki67, and PD-1 to examine their activation status. CD8+ TILs were composed almost entirely of effector memory T cells (mean 75.0%) followed by effector memory RA+ (EMRA) T cells (mean 13.6%), central memory T cells (mean 8.2%) and naïve T cells (mean 3.1%) (Figure 1). This differentiated phenotype of CD8+ TILs suggested an accumulation of antigen experienced T cells. To determine whether this accumulation was driven by reactivity to tumor antigen we next examined CD8+ TILs for signs of recent T cell activation.

To explore potential in situ T cell reactivity to tumor antigen in tumor tissue, CD8+ TILs were examined for various activation markers ex vivo immediately after isolation from tissue. Low frequencies of CD8+ TILs across all samples expressed any activation markers; CD137 (mean 4.0%), OX-40 (mean 1.5%), HLA-DR (mean 1.1%), Ki-67 (mean 6.0%). However, of the 20 tumors analyzed for CD137 expression, we identified 5 tumors with CD137+ frequencies of 5.0% or greater. Strikingly, all tumors surveyed showed high frequencies of PD-1+ CD8+ T cells (mean 68.5%) (Figure 2).

Challenge with autologous tumor cells does not increase the frequency of CD137+ CD8+ TILs

In our previous reports, we described multiple methods employed to identify tumor reactive T cells from either tumor, PBMCs, or TDLNs. We compared tumor-reactivity in breast tumors to melanoma tumors, which are thought to be highly immunogenic, by using a high dose IL-2 method for TIL extraction. In total we screened 7 tumors and 2 tumor-positive TDLNs from 7 breast cancer patients and 6 tumors from 6 melanoma patients (Table 5). In order to prevent PD-1:PD-L1 engagement from inhibiting reactivity, blocking antibodies for PD-1 and PD-L1 were used when sufficient cell numbers allowed for an additional group. In response to autologous tumor challenge, breast TILs had a mean CD137 frequency of 0.61% versus 2.36% in melanoma samples. Addition of PD-1 blockade raised the melanoma CD137 frequency to 3.4%, but only to 0.91% for breast cancer samples (Figure 3C). Measurement of IFN-γ from these cultures confirmed melanoma T cells to be more tumor-reactive with a $p<0.05$ and approaching 0.05 for co-cultures with the addition of PD-1 blocking antibodies respectively (Figure 3D).

Since tumor-reactive T cells may also be found in the PBMCs or TDLNs of patients, we isolated CD8+ T cells from these tissues and co-cultured them with primary tumor cells to examine for reactivity. These samples were analyzed by change in CD137 expression (ΔCD137) as activated non-tumor-specific T cells may easily be found in these compartments. Using 2% ΔCD137 as a threshold, 0/12 patient PBMCs contained tumor-reactive

T cells (Figure 3A), suggesting that they are present at a very low frequency or not present. Of 8 TDLNs surveyed we found two with reactive T cells (Figure 3B), BC81 and BC92. BC81 derived CD8+ T cells increased CD137 expression 1.6% upon culture with tumor cells and 2.1% in the presence of PD-1 blockade. Similarly BC92 derived CD8+ T cells increased CD137 expression 1.0% upon culture with tumor cells and 2.39% in the presence of PD-1 blockade.

CD137+ CD8+ TILs are tumor-reactive

Despite a lack of reactivity in majority of breast tumor CD8+ TILs, select cases of positive reactivity from the above described experiments were further characterized. To do so CD137+ CD8+ T cells were single cell sorted to generate single cell clones. A primary tumor from patient BC166 consisted of CD8+ TILS that were 7.7% CD137+ (Figure 4A). Interestingly, these CD137+ CD8+ TILs were predominantly PD-1^{hi} TIGIT ^{hi} as compared to CD137- CD8+ TILs (Figure 4B). CD137+ CD8+ TILs were expanded ex vivo, rechallenged with autologous tumor cells, and examined again for tumor reactivity by CD137 upregulation. 21.8% of the expanded CD8+ TILs upregulated CD137 in response to tumor challenge, verifying their identity as tumor-reactive (Figure 4C). These bonafide tumor-reactive CD8+ T cells were single cell sorted and expanded as T cell clones.

Sequencing of TCR of tumor reactive T cell clones

As mentioned in our previous report, we amplify Vα and Vβ amplicons separately during the PCR reaction and enrich the product during a second PCR. The amplified product can be visualized by agarose gel electrophoresis where the expected bands are then excised and purified using a gel extraction kit. The purified product is then submitted for Sanger sequencing using T3 or T7 primers to the COH Integrative Genomics Core. The TCR nucleotide sequences are analyzed using IMGT/V-QUEST to determine the identity of the V alpha and V beta of the TCR.

This year we have sequenced the TCR Vβ of 64 CD8 clones from patient BC166 along with some of the TCR Vα regions. Of the 64 clones established, we identified 20 unique clones as determined by T cell receptor (TCR) vBeta CDR3 sequencing (Table 6, Figure 4D). Of these 20 unique clones from BC166, one repeating sequence appeared 27 times, representing the most dominant clone which was able to expand in vitro. Because we have 1 cryopreserved vial of BC166 autologous tumor target remaining, this information minimized the number of clones to be rescreened to confirm T cell reactivity. Additionally, this gave us the sequences for these potential tumor reactive T cell clones, which will be used for further downstream analysis of antigen specificity by the Denver team. For the same reasons as sequencing BC166 clones, we are in the process of sequencing T cell clones previously generated from patients BC81, BC85, and BC87.

Spatial Analysis of CD137+ CD8+ TILs

Whether CD137+ CD8+ TILs could be found in cancer islands of the tumor or near cancer cells was of interest to us. To do so we optimized a TSA IHC staining panel of CD8, CD137, and pan cytokeratin. Using histology, we noticed a membrane polarized staining pattern for CD137 on CD8 T cells (Figure 5A). To determine if this was true staining, we stimulated fresh healthy donor PBMC CD8+ T cells with anti-CD3/CD28 beads overnight, stained with fluorescent antibodies, and prepared cytospins to perform fluorescent microscopy. Indeed, the membrane expression of CD137 on CD8 T cells was polarized demonstrating the unique characteristic of this activation marker (Figure 5B).

We stained tumor tissue from BC166 (a piece resected from fresh tissue) received in the lab that also generated tumor-reactive clones (as described above). Upon analysis of this panel, T cells were defined as being located in 'cancer islands' if the inForm tissue segmentation identified T cells in CK+ cancer islands, 'near cancer cells' if within 25µm of CK+ cancer cells, and in 'stroma' if greater than 25um from CK+ cancer cells. We chose 25µm as a cut-off because size wise it is generally 1-2 cell distances away given that immune cells are generally 7-18µm in size. We found that surprisingly CD137+ CD8+ TILS do not show any significant trend toward being present in the stroma, near cancer, or in cancer islands (Figure 6). To continue this analysis we are in the process of verifying flow cytometry CD137 expression data generated from other tumor samples analyzed using

this IF analysis. We plan on using a set of 'CD137+' tissues to compare to tissues that lack CD137+ CD8 T cells 'CD137-' as a means for understanding what biologically is different between these patients. Potential studies include analysis of regulatory T cells, CD4:CD8 ratios, myeloid cell levels, and mutation load in these patients.

A recent publication demonstrated that a lack of T cell infiltration and anti-tumor responses in metastatic melanoma was correlated to the activation of the WNT/β-catenin signaling pathway in cancer cells [2]. To determine whether the low CD8 T cell responses seen in breast cancer patients was due to β-catenin expression levels on breast cancer cells, we stained 3 'CD137+' and 3 'CD137-' with β-catenin and pan CK. We imaged the slides using our automated Vectra imaging system and quantified β-catenin expression levels on CK+ breast cancer cells using inForm software. The software determines an H-score based on the numbers of cells which fall into each bin scored in a range from 0+ (no staining) to 4+ (bright staining) based on a MFI threshold set by the user (Figure 7A). IF staining revealed that there was no correlation of β-catenin expression levels on breast cancer cells and the frequency of CD8+CD137+ cells determined by flow cytometry (Figure 7B).

Identification of patient derived T cell clones specific for breast cancer cell lines

To determine if tumor-reactive T cell clones target known putative antigens, we screened 22 of the BC166 clones against C1R cells transfected with Her2, CEACAM 5, Mucin 1, Telomerase, and NY-ESO-1 (CTAG) generated by the Slansky team. All of the clones screened did not react against these putative antigens as deemed by CD107 mobilization (Table 7). To determine whether the T cell clones we established could target tumor cells other than their own autologous tumor cells, we reacted T cell clones with several partially HLA class I matched breast cancer cell lines. These cell lines consisted of three ER- cell lines (SKBR3, MDA-MB-231, AU565) and four ER+ cell lines (MCF-7, MCF-7:HER2, HCC1428, CAMA-1). All patient T cell clones used for this assay and all breast cancer cell lines shared expression of the HLA-A02:01 allele, as summarized in Table 8 [3]. CD107 mobilization in greater than or equal to 10% of total CD8s was considered tumor-reactive (Figure 8A, Table 9).

Four BC166 T cell clones (Figure 8B), five BC81 Tumor T cell clones (Figure 8C), one BC85 T cell clone and, two BC87 clones (Figure 8D) demonstrated MHC restricted specificity for shared tumor antigens. One clone from BC166 was identified to be A2 restricted using the MA2.1 HLA-A2 blocking antibody and, therefore the TCR Vβ and Vα sequences were sent to Denver for generation of soluble TCRs and screening against their bacculovirus peptide library. The MHC I restricted clones from BC81, BC85, and BC87 will be screened further to determine if they are HLA-A2 restricted. Finally we demonstrated the ability of several BC166 clones to kill target cancer cells. T cell clones were cultured with target cells for 72h at the given ratio and cell killing was evaluated by fold change of surviving cancer cells (Figure 9A). Live cell imaging confirmed the ability of clones to efficiently target and destroy cancer cells (Figure 9B). We are currently in the process of screening 6 more clones from BC81, 29 clones generated from BC85, 2 clones generated from BC87, and 52 clones generated from BC55 against the 7 breast cancer cell lines to determine if they can target and kill them. We did screen 5 clones from BC86 against the breast cancer cell lines; however, none mobilized CD107 to the degree of true reactivity (Table 9).

Tumor-reactive CD8+ TILs recognize shared non-neoantigens

Specificity of patient derived CD8+ T cell clones for both autologous tumor cells and breast cancer cell lines revealed shared tumor antigens between breast cancer tumors. To evaluate if these antigens were mutational neoantigens, we performed whole exome sequencing based mutational analysis. We sent out DNA from BC166's tumor to the OSHU team to perform whole exome sequencing. The number of identified missense mutations from patient BC166's tumor was 32, which is slightly below average for breast cancer tumors [4]. Thus, elevated mutation load is not the explanation for the elevated T cell reactivity seen in patient BC166. A comparison of the mutations present in BC166 tumor and the various cell lines with shared tumor antigens yielded no identification of shared mutations between them (Table 10). This analysis suggests that T cell clones

with specificity for both autologous tumor and various breast cancer cell lines are likely specific for shared non-neoantigens.

Summary of major findings and plans

- Showed that low frequencies of CD8+ TILs express activation markers upon ex vivo analysis.
- Demonstrated the use of partially HLA matched breast cancer cell lines in identifying tumor-reactive T cells.
- Identified 9 tumor-reactive CD8+ T cell clones from two patients. Four of these have been shown to be A2 restricted and have been TCR sequenced for further antigen identification assays by the UC-Denver team.
- Identified tumor-reactive clones specific for shared antigens (not neoantigens).
- Examined the spatial location of CD137+ CD8+ T cells in tumors.
- Showed that mutational load and β-catenin expression do not explain the high frequency of tumor-reactive T cells seen in BC166 tumor.
- We will continue to screen established clones for reactivity against partially HLA-matched breast cancer cell lines.
- We will use identified patients with a high frequency of CD137+ TILs and a low frequency of CD137+ TILs to understand the biology of why these purported tumor-reactive T cells are or are not present.
- We will continue collaboration with the Slansky lab and embark on a new collaboration with the Pinilla lab at the Torrey Pines Institute to identify the antigen for our established tumor-reactive clones.

Personnel

1. Peter P. Lee, MD – project PI (40% effort)
2. John Yim, MD – CoH Surgical Oncology (5% effort)
3. Joanne Mortimer, MD – CoH Medical Oncology (no salary requested)
4. Tommy Tong, MD – CoH Pathology (no salary requested)
5. Sailesh Pillai, PhD – Assistant Research Professor (no salary requested)
6. Colt Egelston, PhD – post doc (100% effort)
7. Diana Simons – Research Associate II (95% effort)
8. Grace Jimenez – Lab Technician (100% effort)

OVERALL RESEARCH ACCOMPLISHMENTS (YEAR)

KEY RESEARCH ACCOMPLISHMENTS (2013-2014)

- Optimized protocols for using dendritic cell presentation of cancer cell line lysate antigen
- Optimized conditions for successful culture of autologous tumor cells
- Optimized flow cytometry based identification and sorting of antigen-reactive T cells using CD137
- Optimized improved sorting conditions of low numbers of T cells
- Isolated and sent CD8 and CD4 T cells from 16 patients and 96 samples to Denver for TCR repertoire analysis
- Screened 9 patients for tumor reactive T cells and sorted those T cells
- Begun sequencing the TCRs of generated T cell clones
- Begun screening hundreds of generated T cell clones for confirmation of reactivity

KEY RESEARCH ACCOMPLISHMENTS (2014-2015)

- Isolated and sent CD8 and CD4 T cells from 17 patients and 26 samples to Denver for TCR repertoire analysis in 2015
- Screened 34 patients for tumor reactive T cells and sorted reactive T cells for downstream analysis in 2015
- Sequenced the TCRs of generated T cell clones
- Optimized effector and target cell numbers and ratios for T cell cytotoxicity assays.
- Identified that BC patient tumor-T cell reactivity remains unchanged by the addition of PD-1 and PD-L1 blocking antibodies.
- Discovered the majority of CD8 TILs express high levels of PD-1 and TIGIT but do not express other checkpoint molecules, such as LAG-3 and TIM-3, and express low levels of activation markers, CD137 and OX-40, respectively.

KEY RESEARCH ACCOMPLISHMENTS (2015-2016)

- Isolated and sent CD8 and CD4 T cells from 17 patients and 26 samples to Denver for TCR repertoire analysis
- Screened 34 patients for tumor reactive T cells and sorted any reactive T cells for downstream analysis
- Sequenced the TCRs of generated T cell clones
- Optimized effector and target cell numbers and ratios for T cell cytotoxicity assays.
- Identified that BC patient tumor-T cell reactivity remains unchanged by the addition of PD-1 and PD-L1 blocking antibodies.
- Discovered the majority of CD8 TILs express high levels of PD-1 and TIGIT but do not express other checkpoint molecules, such as LAG-3 and TIM-3, and express low levels of activation markers, CD137 and OX-40, respectively.

KEY RESEARCH ACCOMPLISHMENTS (2016)

- Showed that low frequencies of CD8+ TILs express activation markers upon ex vivo analysis.
- Demonstrated the use of partially HLA matched breast cancer cell lines in identifying tumor-reactive T cells.
- Identified 9 tumor-reactive CD8+ T cell clones from two patients. Four of these have been shown to be A2 restricted and have been TCR sequenced for further antigen identification assays by the UC-Denver team.
- Identified tumor-reactive clones specific for shared non-neoantigens.
- Examined the spatial location of CD137+ CD8+ T cells in tumors.
- Showed that mutational load and β-catenin expression do not explain the high frequency of tumor-reactive T cells seen in BC166 tumor.

REPORTABLE OUTCOMES

1. Egelston C, Simons DL, Pillai SG, Jimenez G, Avalos C, Butler TM, Rozanov D, Spellman PT, Melstrom LG, Jung JY, Yuan Y, Yim JH, Lee PP. Identification of tumor-reactive CD8+ T cells in ER+ human breast tumors. Submitted to JCI December 2016.
2. Munson DJ, Egelston CA, Chiotti KE, et al., Identification of shared TCR sequences from T cells in human breast cancer using emulsion RT-PCR. PNAS, 2016 Jul 19; 113(29):8272-7.

CONCLUSION:

Over the last four years, our group has worked to understand the frequency and specificity of native tumor-reactive T cells in breast cancer patients. To do so we have accrued a number of patients and studied at great depth T cells from tumor, peripheral blood, and tumor draining lymph nodes. Our patient set largely consisted of patients with ER+ HER-2- tumors. This allowed us to study the tumor-reactive response of T cells in patients where a clear prognostic advantage of tumor infiltrating T cells is not clear [5-7] and where HER-2 antigen elicited T cells are unlikely.

We have made great strides in characterizing CD8+ T cell tumor-reactive responses in the last several years. Using a stream of patient samples contributed by us, paired TCR sequencing led by the Slansky team has yielded the identification of shared TCRs in HLA matched breast cancer patients. This work suggests shared antigens driving a common immune response in breast cancer patients. On our end, we have screened numerous patients for either signs of in situ activation by high dimensional phenotyping of patient T cells or by tumor-reactivity experiments ex vivo. Attempts at numerous strategies over the years led us to settle on using autologous tumor cells as targets and CD137 expression as a readout for tumor-reactive T cells. While we have found that in general tumor-reactive T cells are of a low frequency in the ER+ breast cancer patients we surveyed, we did identify several patients with tumor-reactive T cells. In one patient where we found a high frequency of tumor-reactive CD8+ T cells, we showed with the help of the Spellman team that this high frequency was not due to mutation load or reactivity to neoantigens. Continued work in the very near future will allow us to identify the antigens that our established tumor-reactive clones are specific for and to understand the biology of why a subset of ER+ patients has tumor-reactive T cells. Answers to both of these questions will have significant therapeutic impact.

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7. Ali, H.R., et al., *Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients.* Ann Oncol, 2014. **25**(8): p. 1536-43.

APPENDICES:
None at this time

SUPPORTING DATA:

Table 1. Clinical characterisitics of breast cancer patients enrolled into this study since October 2015

ID#	Age	TMR Type	LN T+/T-	Therapy	Stage	TMR Grade	ER	PR	HER2	SUBTYPE
68	64	I/D	T-	N	Ia	II	+	+	-	LUM
75	53	R:I/D/C L:L		N	Ia	II	+	+	-	LUM
98	50	I/D		N	IIa	II	+	+	-	LUM
126	42	I/D		N	IIIc	II	+	-	-	LUM
130	55	I/D	T+	N	IIb	II	+	+	-	LUM
134	31	I		N	IIb	II	+	+	-	LUM
141	70	I/L/D/C		N	IIa	II	+	-	+	LUM
144	29	I/D		Y	IIb	II	+	-	-	LUM
158	46	I/D	T+	N	IIIc	I	+	+	-	LUM
165	32	I/D		N	Ia	II	+	+	+	LUM
167	58	I/D		N	IIIa	III	+	+	-	LUM
186	55	I/D		N	IIa	II	+	-	-	LUM
187	53	I/D		N	IIIa	I	+	+	-	LUM
192	64	I/D		N	IIa	III	+	+	+	LUM
193	93	L/C		N	Ia	II	+	+	-	LUM
197	49	L:P/I/D R:D		N	Ia	I	+	+	-	LUM
213	47	L:D R:I/D		N	Ia	II	+	-	-	LUM
214	46	I/D		N	Ia	III	+	+	-	LUM
215	31	I/D		N	IIa	III	+	+	-	LUM
233	39	I/D		N	IIa	III	+	+	-	LUM
244	70	L		N	IIa	III	+	+	-	LUM
255	57	L		N	Ia	II	+	+	-	LUM
258	47	I		N	IIa	III	+	-	+	LUM
262	71	I		N	Ia	II	+	+	-	LUM
271	49	I		N	IIb	R-II; L-II	+	+	-	LUM

For Tumor Type: I=invasive ductal carcinoma, D= ductal carcinoma in situ, L= invasive lobular carcinoma, C= lobular carcinoma in situ, P= papillary carcinoma. R and L indicate right and left tumor if applicable. Patient BC144 completed neoadjuvant 8 weeks before surgery. TDLN tumor invasion status is shown for TDLNs used for this study: tumor positive (T+) or tumor negative (T-).

Table 2. Samples sent for HLA-A and -DRB1 typing since beginning of study		
Patient	**HLA-A**	**HLA-DRB1**
A	02:06 31:01	04:07
B	01:01 24:02	04:02 11:01
C	02:01 24:02	12:02 15:02
D	02:01 29:02	07:01 13:01
e	02:01 26:01	01:01 03:01
1	02:01 03:01	01:01 15:01
2	01:01 02:01	03:01 07:01
3	02:01 32:01	01:01 15:01
4	02:01	13:03 15:01
54	02:01	04:01 15:01
55	02:01 03:01	12:01 13:01
57	01:01 02:01	03:01 07:01
61	02:02 30:02	03:01 07:01
67	01:01 02:01	03:01 04:04
70	02:01 32:02	04:04 15:01
71	02:01	07:01
72	02:03 03:01	04:05 13:06
75	02:01 03:01	04:01 15:01
80	02:06 33:01	04:07 11:05
81	01:01 02:01	04:01 13:01
85	02:01 03:01	04:03 11:02
86	02:01 31:01	08:01 14:04
87	02:06 74:01	04:07 07:01
92	02:06 74:01	04:07 07:01
94	02:01 23:01	03:01 04:01
96	02:01 03:01	1:01 13:03
98	02:01 32:01	04:01 13:01
107	02:06 33:03	13:02 15:01
113	01:01 02:01	03:01 11:01
118	02:01 24:02	04:07 14:06
124	02:01 03:01	04:03 16:01
132	02:01 24:02	07:01
166	02:01 02:06	04:03 14:02

Table 3. Complete HLA typing of breast cancer patients						
BC81	**A*01:01**	**A*02:01**	**B*08:01**	**B*44:02**	**C*05:01**	**C*07:01**
BC85	**A*02:01**	**A*03:01**	**B*27:02**	**B*44:02**	**C*02:02**	**C*05:01**
BC87	**A*02:06**	**A*74:01**	**B*39:05**	**B*44:03**	**C*04:01**	**C*07:02**
BC166	**A*02:01**	**A*02:06**	**B*27:05**	**B*35:17**	**C*02:02**	**C*04:01**

Table 4. Clinical characterisitics of breast cancer patients utilized for phenotyping and evaluating T cell activation

ID#	Age	TMR Type	LN T+/T-	Therapy	Stage	TMR Grade	ER	PR	HER2
61	57	I/D	T-	N	IIa	III	+	+	-
68	64	I/D	T-	N	Ia	II	+	+	-
75	53	R:I/D/C L:L		N	Ia	II	+	+	-
80	29	I/D	T-	N	IIa	III	+	+	-
81	70	I/D	T+	N	IIa	II	+	+	-
85	71	I	T-	N	IIa	III	+	-	-
86	35	I/D	T-	N	Ia	III	+	+	-
87	42	I/D	T+	N	IIIa	III	+	+	-
92	53	I	T-	N	Ia	II	+	+	-
94	50	L/C		N	Ia	II	+	+	-
96	45	I/D		N	IIa	II	+	+	-
98	50	I/D		N	IIa	II	+	+	-
118	77	I/D		N	IIa	III	+	+	-
126	42	I/D		N	IIIc	II	+	-	-
130	55	I/D	T+	N	IIb	II	+	+	-
134	31	I		N	IIb	II	+	+	-
141	70	I/L/D/C		N	IIa	II	+	-	+
144	29	I/D		Y	IIb	II	+	-	-
158	46	I/D	T+	N	IIIc	I	+	+	-
164	64	R:I/D L:I/D		N	IIIa	R-II; L-III	+/+	+/+	-/-
165	32	I/D		N	Ia	II	+	+	+
166	35	I		N	IIa	II	+	+	+
167	58	I/D		N	IIIa	III	+	+	-
186	55	I/D		N	IIa	II	+	-	-
187	53	I/D		N	IIIa	I	+	+	-
192	64	I/D		N	IIa	III	+	+	+
193	93	L/C		N	Ia	II	+	+	-
197	49	L:P/I/D R:D		N	Ia	I	+	+	-
213	47	L:D R:I/D		N	Ia	II	+	-	-
214	46	I/D		N	Ia	III	+	+	-
215	31	I/D		N	IIa	III	+	+	-
233	39	I/D		N	IIa	III	+	+	-
244	70	L		N	IIa	III	+	+	-
255	57	L		N	Ia	II	+	+	-
258	47	I		N	IIa	III	+	-	+
262	71	I		N	Ia	II	+	+	-
271	49	I		N	IIb	R-II; L-II	+	+	-

For Tumor Type: I=invasive ductal carcinoma, D= ductal carcinoma in situ, L= invasive lobular carcinoma, C= lobular carcinoma in situ, P= papillary carcinoma. R and L indicate right and left tumor if applicable. Patient BC144 completed neoadjuvant 8 weeks before surgery. TDLN tumor invasion status is shown for TDLNs used for this study: tumor positive (T+) or tumor negative (T-).

Table 5. Clinical characteristics of melanoma patients in this study.

ID	Age	Gender	Therapy	Primary/met	Reccurrence
Mel01	56	M	N	Primary	N
Mel02	79	M	N	Primary site	Y
Mel03	92	F	N	Primary	N
Mel04	64	M	N	Met	Y
Mel05	33	M	N	Nodal met	N
Mel06	67	F	N	Nodal met	Y

Table 6. TCR vBeta CDR3 sequences of BC166 clones.

Clone ID	# clones	Vbeta CDR3	Vbeta Family
A	1	CSARDPTSGTADTQFF	20-1
B	27	CAWSGPVGGVAHNEQFF	30
C	3	CASSPVRRGIMNTEAFF	27
D	2	CSAETGAQYF	20-1
E	1	CTSSQVPGQGDEQYF	4-1
F	1	CSAPTSGRFTGELFF	20-1
G	1	CTSSQVPGHGDEQYF	4-1
H	9	CASSFNQLWNEQFF	5-4
I	3	CATSSGTGVQPQHF	15
J	1	CASSQVTGRQPQHF	4-1
K	1	CAWSGPVGGVALNEQFF	30
L	1	CASSFSTCSANYGYPF	12-3
M	1	CASSGQVNEQFF	5-4
N	1	CASGFGGAGGYTF	25-1
O	3	CASREGPGPTTEAFF	5-1
P	1	CASSFAGGNTEAFF	12-3
Q*	1	CSAETGAQYF	20-1
R	1	CASSQVPGHGDEQYF	4-1
S*	1	CAWSGPVGGVAHNEQFF	30
T*	1	CASSLGAWRGM##GYTF	27

* Denotes an unproductive sequence prediction by IMGT, which does not affect their identification as unique.

Table 7. Δ %CD107 of CD8 from BC166 clones screened against known putative antigens transfected into C1R cells

	stim-max(unstim or C1R:A2)				
Clone #	**Her2**	**CEA**	**CTAG**	**MUC-1**	**Telo**
1	1.29	-1.30	-1.61	-1.54	-1.40
33	-1.11	-3.23	-1.95	-2.57	0.95
27	0.26	-1.51	-1.91	-1.81	-1.80
62	-1.72	-1.96	-2.04	-2.05	-2.21
34	-1.18	-1.38	-1.44	-1.48	-1.44
27	0.20	0.01	0.54	-0.08	-0.04
37	-0.27	-1.12	-0.39	-0.55	-1.28
14	-0.04	-0.18	-0.33	-0.14	-0.26
40	-1.13	-0.97	-1.16	-1.05	-1.16
47	0.09	-0.16	0.01	-0.08	-0.26
43	-0.80	-0.68	-0.80	-0.89	-0.73
51	0.33	-0.38	-0.29	-0.43	-0.32
31	1.16	1.03	1.00	0.33	2.23
12	-0.13	0.06	-0.22	-0.19	-0.22
15	-0.05	0.06	-0.10	-0.08	0.02
49	-0.13	-0.09	-0.17	-0.15	-0.20
53	-0.44	-0.28	-0.25	-0.34	-0.33
54	-0.15	-0.14	-0.24	-0.13	-0.02
56	0.13	-0.30	-0.43	-0.21	-0.08
64	1.34	0.85	-0.15	0.23	0.44
26	-0.57	-0.46	-0.30	-0.48	-0.67
17	0.13	0.46	0.29	0.27	0.26

Table 8. HLA phenotyping of breast cancer cell lines.

AU565	A*02:01	A*03:01	B*14:01	B*40:01	C*03:04	C*08:02
CAMA-1	A*02:01	A*32:01	B*40:02	B*15:01	C*02:02	C*03:03
HCC1428	A*01:01	A*02:01	B*07:02	B*07:02	C*07:02	C*07:02
MDA-MB-231	A*02:17	A*02:01	B*41:01	B*40:02	C*17:01	C*02:02
MCF-7	A*02:01	A*02:01	B*18:01	B*44:02	C*05:01	C*05:01
SKBR3	A*02:01	A*02:01	B*14:02	B*39/B*50/B*55	C*03:04	C*03:04

Table 9. Breast cancer patients T cell clones screened against breast cancer cell lines. Values are Δ %CD107 of CD8s.

BC166

Clone ID	AU-565	CAMA-1	HCC1428	MB231	MCF-7	MCF-7/HER	SKBR3
A	0.35	2.24	0.77	0.81	0.66	0.25	0.52
B	0.01	2.58	3.56	0.92	2.91	0.73	0.19
C	1.32	**85.89**	**29.99**	0.48	0.86	0.37	3.08
D	**23.76**	1.83	1.75	0.24	0.65	0.98	0.32
E	0.38	0.84	1.57	0.27	1.19	0.66	0.16
F	0.12	0.24	0.26	0.15	0.75	0.43	0.27
G	0.40	2.97	3.47	0.64	1.70	0.96	0.28
H	0.60	1.35	5.18	1.58	3.38	3.69	0.46
I	1.33	3.86	7.00	2.66	2.41	1.41	0.84
J	0.15	1.39	3.45	0.13	0.24	0.11	0.64
K	-0.18	0.24	4.54	-0.08	-0.09	-0.05	-0.01
L	0.58	2.34	4.32	2.02	2.64	2.56	0.69
M	0.27	2.03	2.20	1.87	**10.72**	0.84	0.34
N	0.13	0.37	1.16	2.33	0.22	-0.01	0.11
O	0.27	0.36	1.08	0.42	0.73	0.54	0.21
P	0.69	2.94	4.53	2.11	3.16	0.77	0.49
Q	0.26	0.16	0.91	0.10	0.60	0.48	0.18
R	0.56	1.09	2.00	0.66	1.02	1.44	0.42
S	-0.70	**81.09**	**30.09**	1.00	0.00	0.03	0.77
T	0.44	0.99	1.18	0.43	0.93	1.35	0.15

BC81

Clone ID	AU-565	CAMA-1	HCC1428	MB231	MCF-7	MCF-7/HER	SKBR3
1	0.96	1.71	1.86	3.25	1.03	0.39	1.01
2	1.07	1.18	1.34	3.03	1.38	1.43	1.36
3	4.16	6.70	2.56	6.65	**12.06**	3.05	1.62
5	1.45	1.57	1.44	4.18	2.82	1.77	
6	0.70	1.70	1.57	3.02	0.85	0.20	0.63
7	1.78	1.03	2.65	3.82	5.08	1.67	0.96
8	-0.20	1.27	1.21	1.72	0.24	0.07	0.18
9	2.15	2.36	1.40	3.09	2.14	1.21	1.39
10	0.26	0.08	-0.55	2.11	-0.43	0.16	-0.42
11	-1.49	-2.35	-1.79	-2.22	-1.18	3.19	-1.26
12	1.12	1.00	1.42	4.70	1.02	1.20	0.68
13	0.62	1.38	1.33	2.65	0.77	0.45	0.48
14	0.37	2.27	3.77	2.32	1.04	-0.32	-0.04
17	1.16	1.79	1.22	4.23	1.66	0.73	0.99
18	0.51	1.64	1.70	2.88	0.67	0.18	0.40
19	-2.02	-2.39	-2.31	-1.67	7.02	5.07	-2.00
22	1.16	2.10	1.23	4.14	2.58	1.41	1.00
23	-1.90	-2.06	-0.94	-1.14	-1.33	-1.30	-1.79
26	-1.75	-1.24	0.19	-1.29	-1.01	-1.35	-1.92
27	0.69	3.33	1.44	2.57	4.75	2.68	1.17
29	1.09	1.74	1.17	**31.76**	2.04	5.04	1.10
31	0.97	1.94	1.75	6.57	1.00	0.85	0.95
34	1.80	1.63	2.35	5.75	6.58	0.53	1.23
37	1.26	1.60	2.33	5.40	2.57	1.14	1.44
39	0.14	0.82	1.64	3.56	5.25	3.12	0.77
40	0.92	1.08	0.60	2.84	1.00	0.33	0.60
41	0.12	1.45	1.83	3.64	0.50	0.21	0.31
43	1.77	2.17	3.72	2.42	0.83	0.31	0.60
44	0.99	2.04	1.55	2.38	0.82	1.07	1.19
49	0.65	0.71	0.89	3.67	0.78	0.21	0.59
50	3.55	**14.09**	3.30	6.50	2.55	4.27	3.59
54	0.35	0.25	0.92	0.72	0.48	-0.62	-0.15
55	0.32	0.28	0.34	0.60	**44.36**	**31.66**	0.18
56	-1.78	-1.04	-0.63	2.56	-0.13	-1.37	-1.68
57	4.29	5.48	1.63	7.44	**16.70**	2.56	2.44
60	1.00	2.96	0.85	3.07	1.56	0.86	0.33

BC85

Clone ID	AU-565	CAMA-1	HCC1428	MB231	MCF-7	MCF-7/HER	SKBR3
BC85.11	-6.25	8.99	-2.75	-5.56	5.59	-5.11	-4.74
BC85.14	-0.25	0.19	2.81	0.80	1.95	-0.02	-0.37
BC85.18	0.32	0.89	2.94	1.80	7.89	3.63	4.24
BC85.2	0.76	1.10	7.41	1.79	2.71	4.66	4.25
BC85.28	-1.93	-1.26	-0.29	-1.05	7.27	4.43	7.57
BC85.32	-3.56	-3.60	-1.59	-0.42	2.18	0.87	5.48
BC85.34	0.30	0.68	2.20	0.83	7.30	5.09	8.01
BC85.36	0.66	1.04	3.11	1.61	5.71	2.33	2.07
BC85.38	0.61	1.38	4.90	2.92	3.72	1.53	0.66
BC85.39	0.18	1.72	3.69	2.32	10.69	3.89	7.72
BC85.6	-0.65	0.02	1.93	0.45	3.59	-0.01	-0.12
BC85.9	0.24	0.24	2.25	1.23	1.57	0.15	-0.19
BC85.16	-0.01	0.04	2.27	0.45	1.57	0.11	0.09
BC85.20	-5.02	-4.97	-3.61	-4.86	-3.76	-5.18	-4.53
BC85.5	-1.18	-1.11	-0.88	-0.65	-0.68	-0.82	
BC85.23	-3.70	-2.60	-5.10	-8.10	-0.30	-3.60	
BC85.27	0.61	0.08	0.40	0.35	5.00	2.37	0.54
BC85.37	-7.32	2.40	-4.37	-8.48	-0.30	-7.12	
BC85.21	0.29	0.37	0.56	0.71	1.39	0.54	

BC86

Clone ID	AU-565	CAMA-1	HCC1428	MB231	MCF-7	MCF-7/HER	SKBR3
BC86.1	0.07	-0.05	3.97	1.70	8.45	-0.08	1.29
BC86.2	-0.08	0.39	1.89	0.39	6.28	0.33	-0.04
BC86.3	0.76	1.12	4.18	2.22	4.11	1.46	0.91
BC86.4	0.30	0.64	2.40	1.07	3.61	0.37	0.05
BC86.5	0.01	0.75	6.44	2.39	1.68	1.38	0.45

BC87

Clone ID	AU-565	CAMA-1	HCC1428	MB231	MCF-7	MCF-7/HER	SKBR3
BC87.1	-0.43	12.35	0.93	-0.05	2.07	2.79	-0.09
BC87.4	1.39	7.78	2.83	9.22	7.04	4.85	3.96

Table 10. Missense mutations identified in patient BC166. Missense mutations identified in patient BC166 are shown by gene symbol and the resulting protein change. Target cell lines with mutations in the same gene are shown as well as the resulting change.

Gene Symbol	Patient Protein Change	Target Protein Change	Cell Line
ADRM1	p.P242R	-	-
AOC3	p.A275V	-	-
AQP10	p.R17H	-	-
C7orf10	p.A209S	-	-
CCDC28B	p.V27I	-	-
CCDC61	p.V20L	-	-
CCDC80	p.P114L	-	-
CNTN3	p.T313I	-	-
COQ6	p.P347R	-	-
CRYBG3	p.P1248T	p.E774*	HCC1428
CSPP1	p.V542I	-	-
DDX24	p.A80P	-	-
DENND2C	p.R570Q	-	-
ENAH	p.G376V	p.E115G	HCC1428
GOLGA6A	p.V495M	-	-
ITGA11	p.G171V	-	-
KIAA1551	p.V1536G	p.K600R	HCC1428
MUC22	p.T1187K	-	-
NAA15	p.Q147H	-	-
NLRP10	p.T151M	-	-
OAS3	p.F430L	-	-
OVGP1	p.D137H	p.S531T, pS595T	CAMA-1
PIK3CA	p.F960V	-	-
POTEM	p.S112G	-	-
PURA	p.L180P	-	-
STAT4	p.S702L	-	-
SUGP2	p.A533V	-	-
SYNE1	p.K7092T	p.D4497H, p.Q3070L, p.E7770*, p.D4750H, p.Q3102L, p.E7453*, p.Q3063L, p.E7841*, p.D4679H	AU565
TTLL6	p.R290H	-	-
UPF3B	p.E383K	-	-
ZFPM2	p.K1139M	-	-
ZNF667	p.R454H	-	-

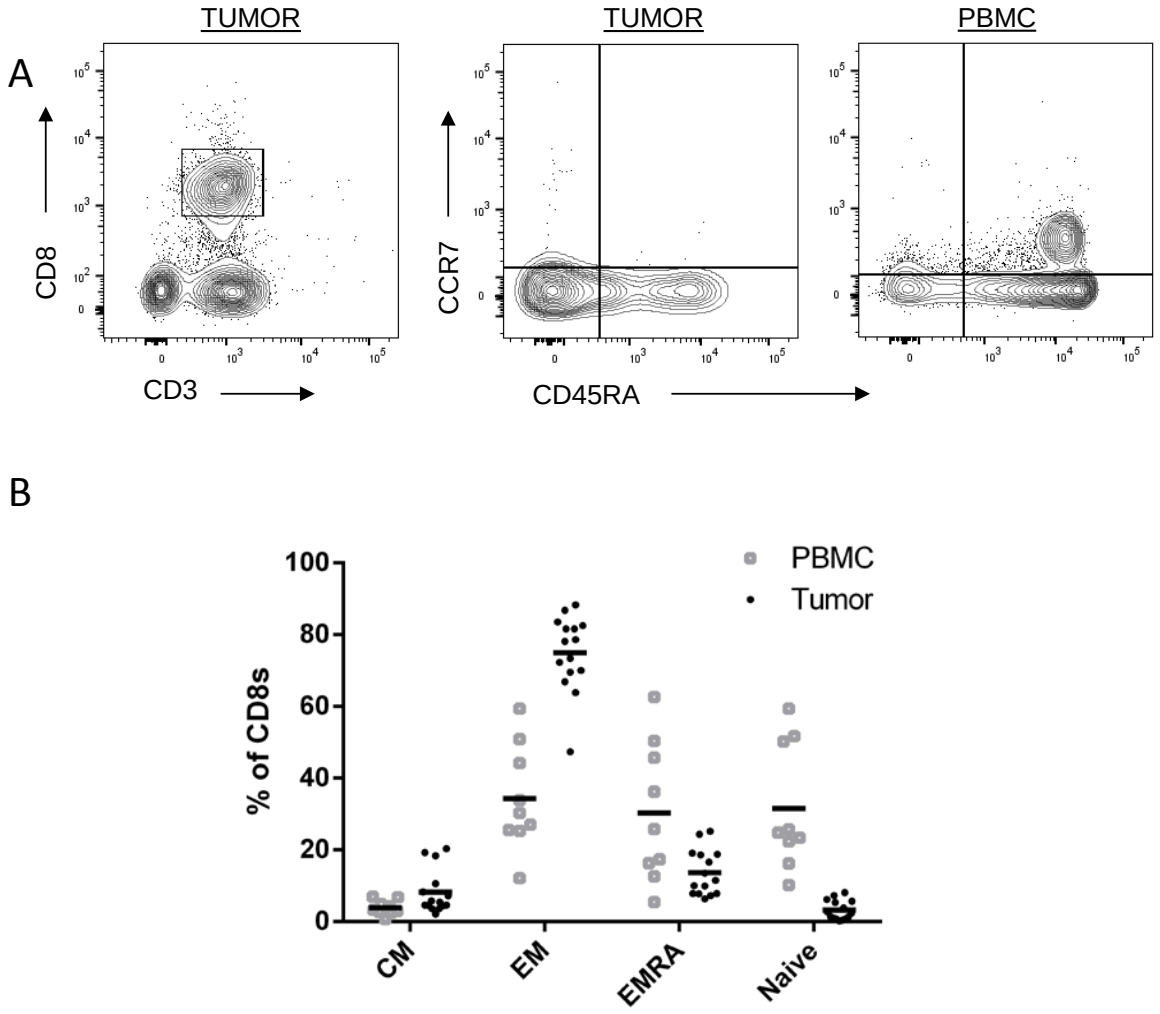


Figure 1. CD8+ TILs in breast cancer patients are antigen experienced and primarily effector memory T cells. Single cell suspensions of tumor and PBMCs were analyzed by flow cytometry for markers of differentiated CD8+ T cell subsets: Naïve (CD45RA+ CCR7+); Central Memory (CD45RA-, CCR7+); Effector Memory (CD45RA-, CCR7-); Effector Memory RA+ (CD45RA+, CCR7-). Representative flow plots (A) and summary of data (B) are shown.

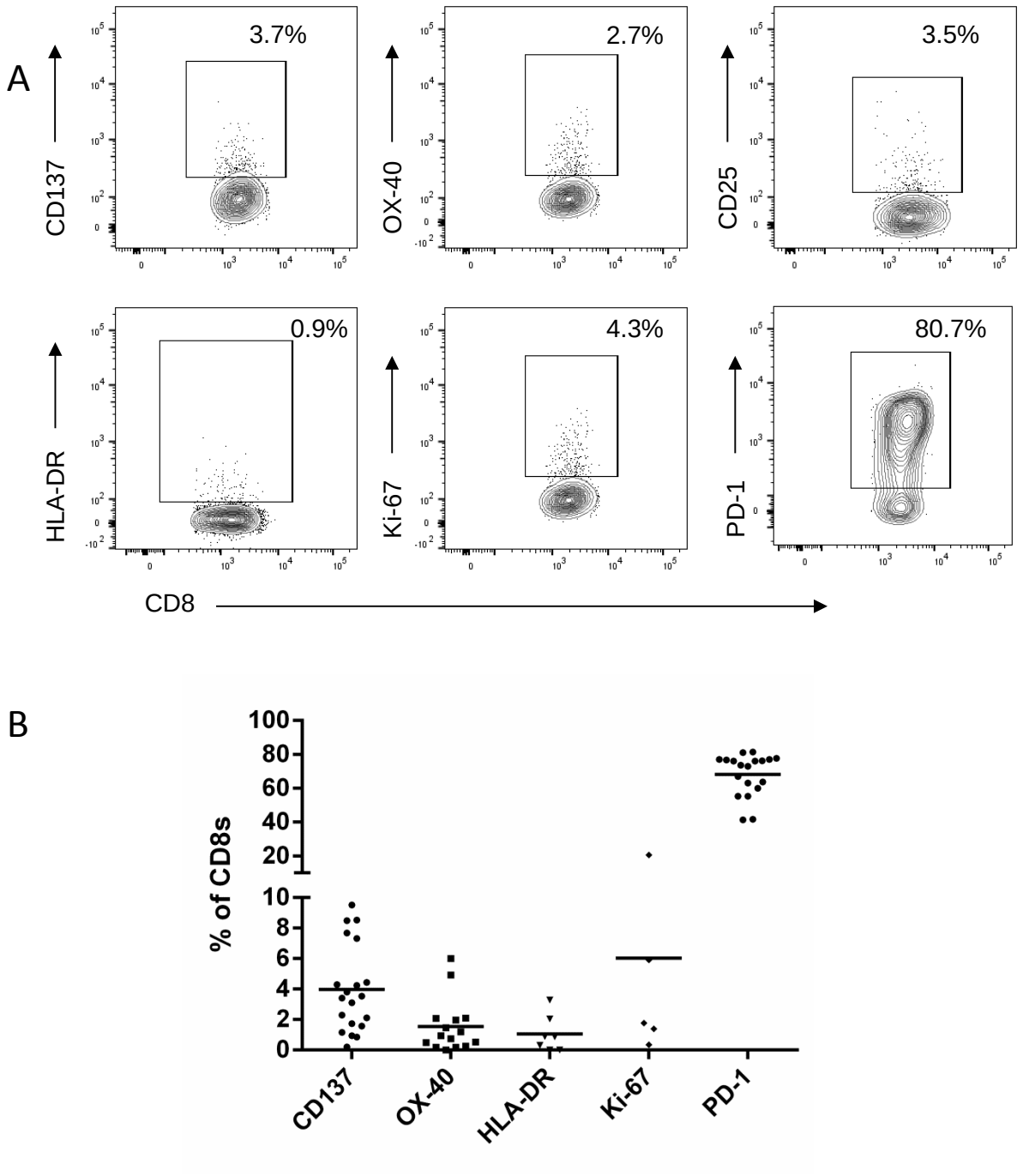


Figure 2. A high percentages of CD8+ TILs express PD-1 but a low percentage express various activation markers. Single cell suspensions of breast tumors were immediately stained and analyzed by flow cytometry for PD-1 and various markers of T cell activation, including CD137, OX-40, HLA-DR, and Ki-67. Representative flow plots (A) and summary of data (B) are shown.

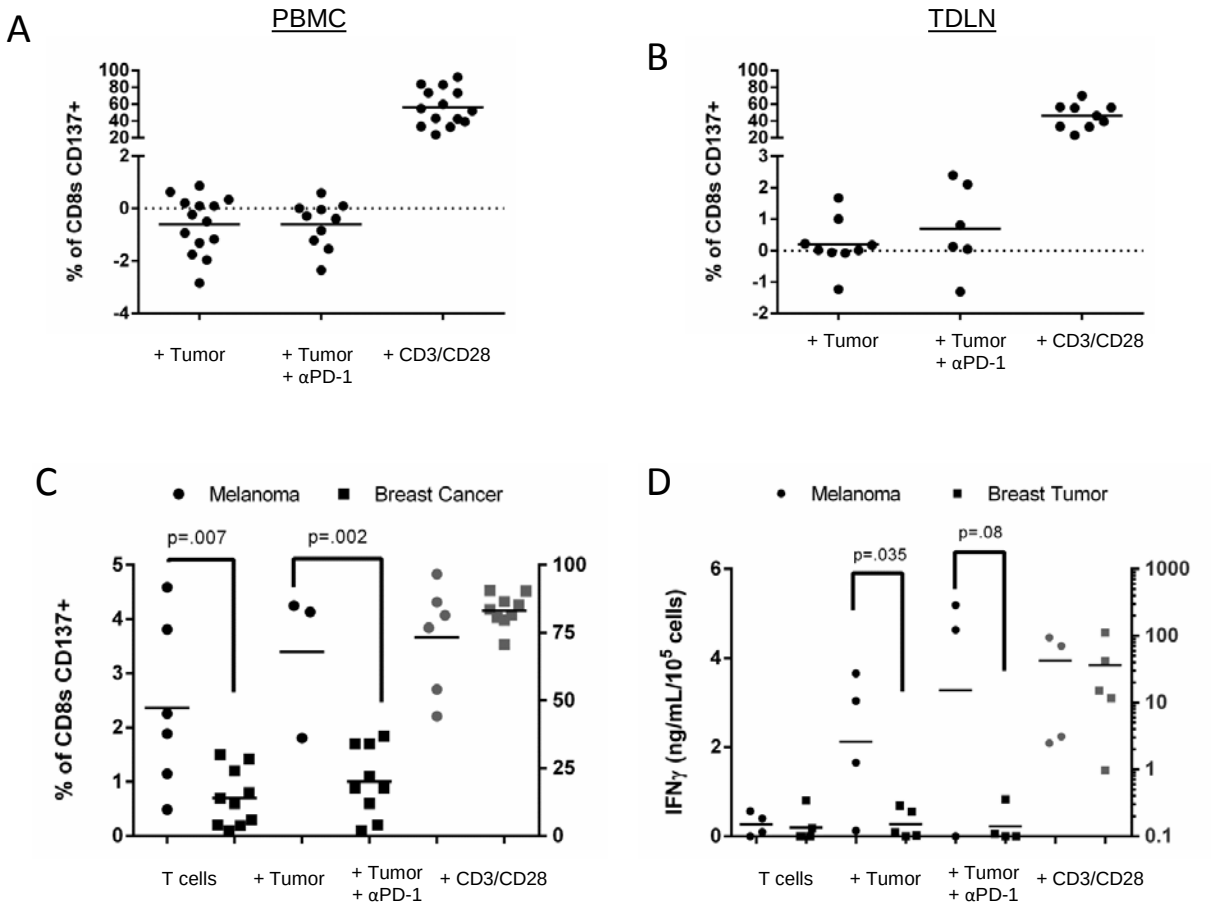


Figure 3. A low percentage of breast cancer patients have tumor reactive T cells at a detectable level. CD8+ T cells isolated from patient PBMCs (A), SLNs (B), or tumor (C, D) were co-cultured with autologous tumor cells and examined for reactivity by upregulation of CD137 (C) or IFN-γ secretion (D). For C and D, CD3/CD28 stimulated conditions shown in gray are graphed on the right axis.

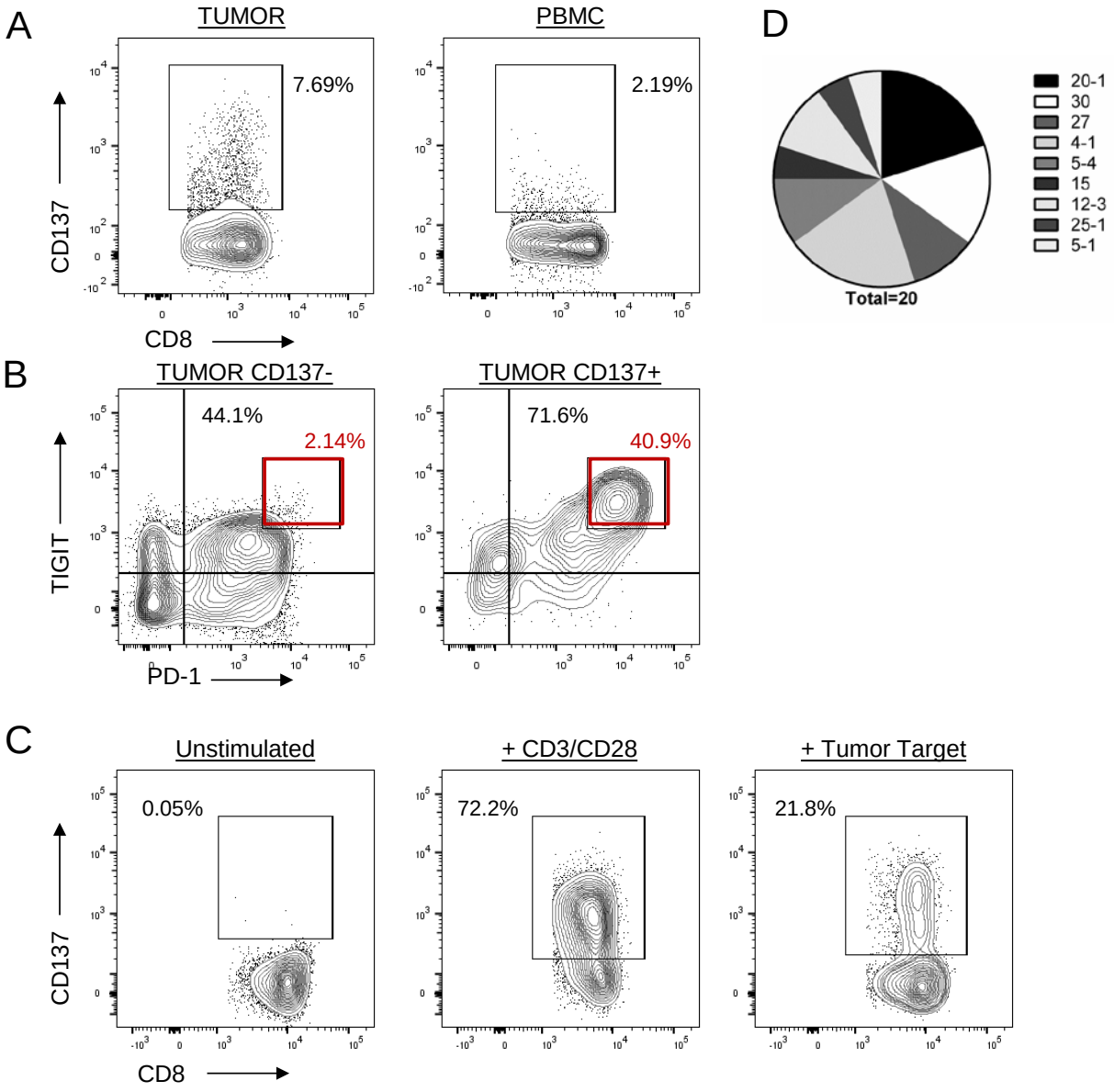
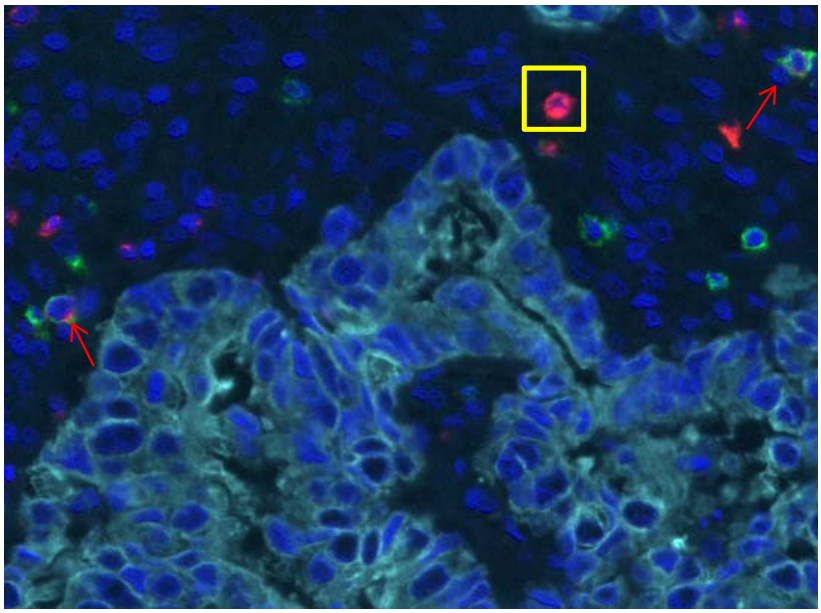


Figure 4. Patient BC166 contains a significant portion of tumor reactive CD8+ TILs. Patient BC166 tumor contained a robust population of CD137+ CD8+ T cells. PBMC CD8+ T cells from the same patient are shown for reference (A). Tumor CD137+ CD8+ T cells are enriched for PD-1^{hi} $TIGIT^{hi}$ cells (B). Expanded T cells from the original CD137+ population upregulated CD137 again when restimulated with autologous tumor cells (C). 20 unique single cell clones were established from the reactive clones as measured by sequencing of the TCR Vbeta CDR3. Vbeta families of each clone are shown (D).

A



B

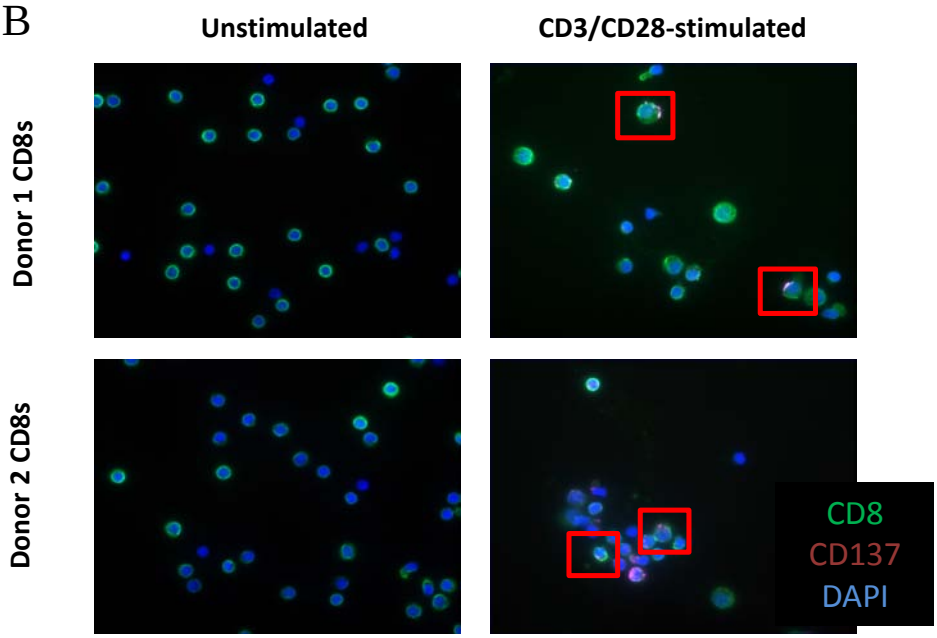


Figure 5. CD137 membrane polarization on CD8 T cells. Representative image of FFPE breast tumor tissue stained with CD8 Opal520-green, CD137 Opal620-magenta, and AE1/AE3 pan cytokeratin Opal690-cyan to determine where CD137+CD8+ TILs are spatially located, the cancer islands of the tumor or near cancer cells. The red arrows point to the CD137+CD8+ TILs and the yellow box surrounds a CD8- cell (A). Cytospins were prepared from healthy donor PBMCs stimulated overnight with anti-CD3/CD28 beads or remained unstimulated, stained with fluorescent antibodies CD8 FITC, CD137 APC, and DAPI for fluorescent microscopy (B). The red boxes highlight CD137+CD8+ double positive T cells.

Figure 6. Spatial distribution of CD8+ TILs from patient BC166. CD137+ and CD137- CD8 TILs were quantified in patient BC166 tumor using a Vectra Imaging System and Inform software. After tissue segmentation T cells were regarded as in tumor if found present in cancer islands, as near tumor if within 25µm of a cancer cell, and in stroma if not within 25 µm of a cancer cell.

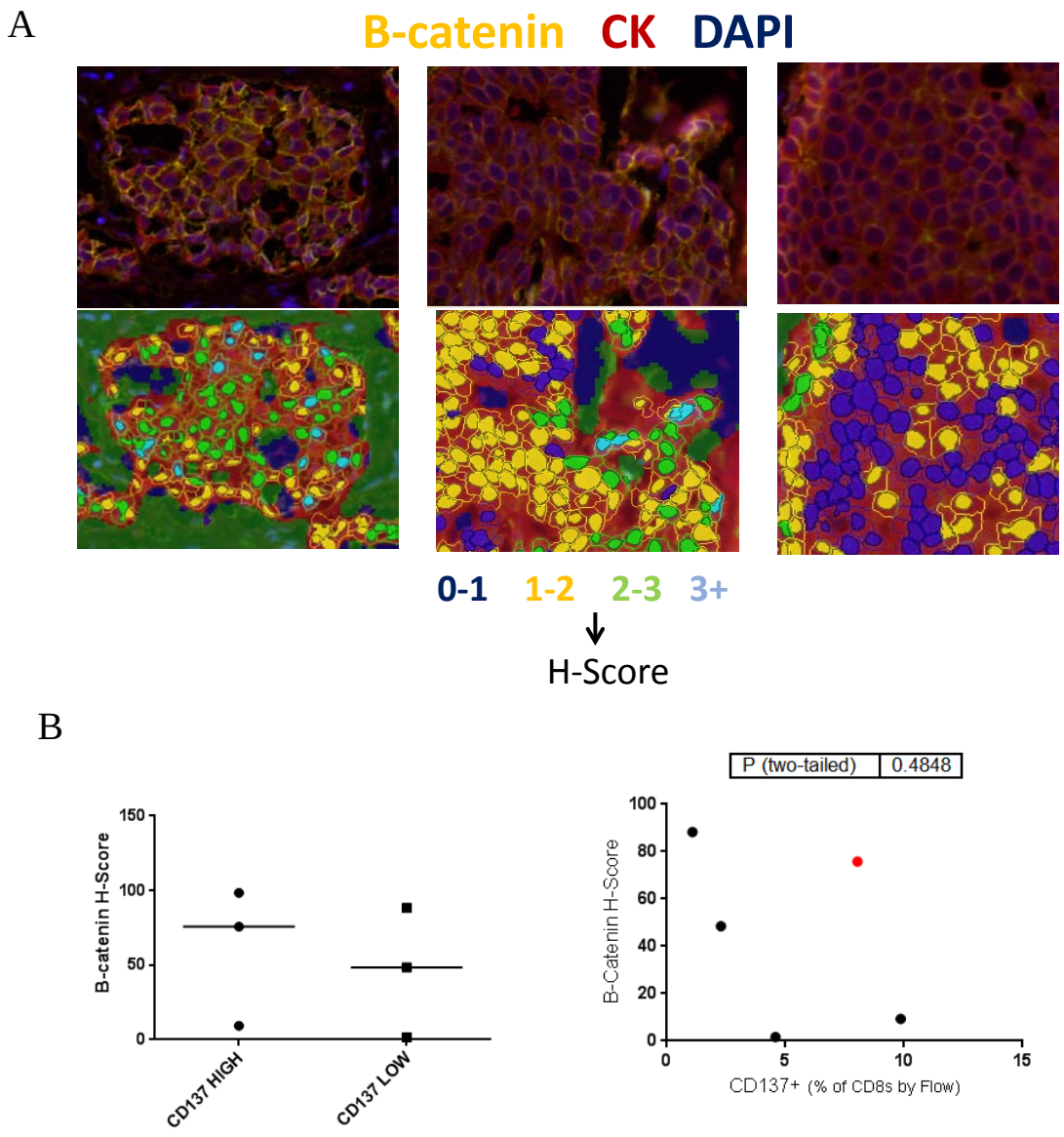


Figure 7. β-catenin expression on cancer cells does not correlate with the frequency of CD137+CD8+ TILs. IF staining of FFPE breast tumor specimens for β-catenin Opal570 (yellow), pan-CK Opal620 (red), and counterstain DAPI (blue) to determine the in vivo expression levels of β-catenin on cancer cells (upper images; A). inForm analysis was able to bin the MFI expression (0+ to 3+) based on the intensity of the fluorescent stain and generate an H-score (lower images; A). The H-score of β-catenin expression levels did not correlate with breast cancer specimens containing high numbers of CD137+CD8+, as determined by flow cytometry (B).

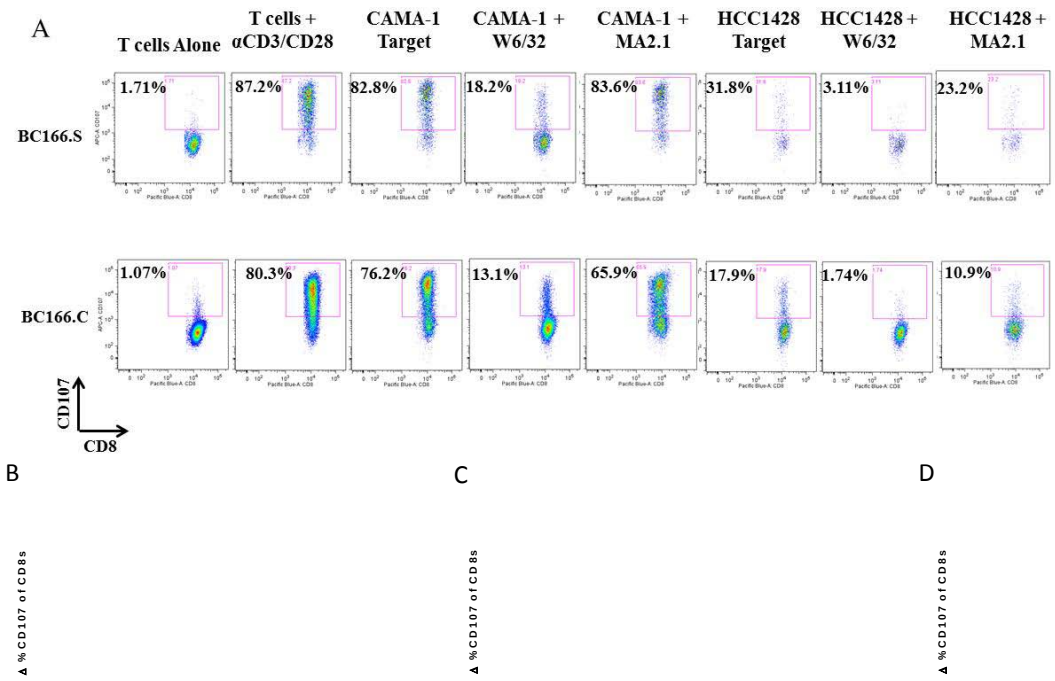


Figure 8. Various patient clones can target breast cancer cell lines in an MHC-restricted fashion. T cell clones were assayed for cancer cell specificity by co-culture with breast cancer cell lines followed by a CD107 mobilization assay. MHC restriction and HLA-A2 restriction of T cell clones was verified by addition of the pan-MHC I blocking antibody W6/32 and MA2.1, respectively, during co-culture. Representative plots are shown (A). Summary data and MHC restriction results are shown for BC166 clones (B), BC81 clones (C) and, BC85 and BC87 clones (D).

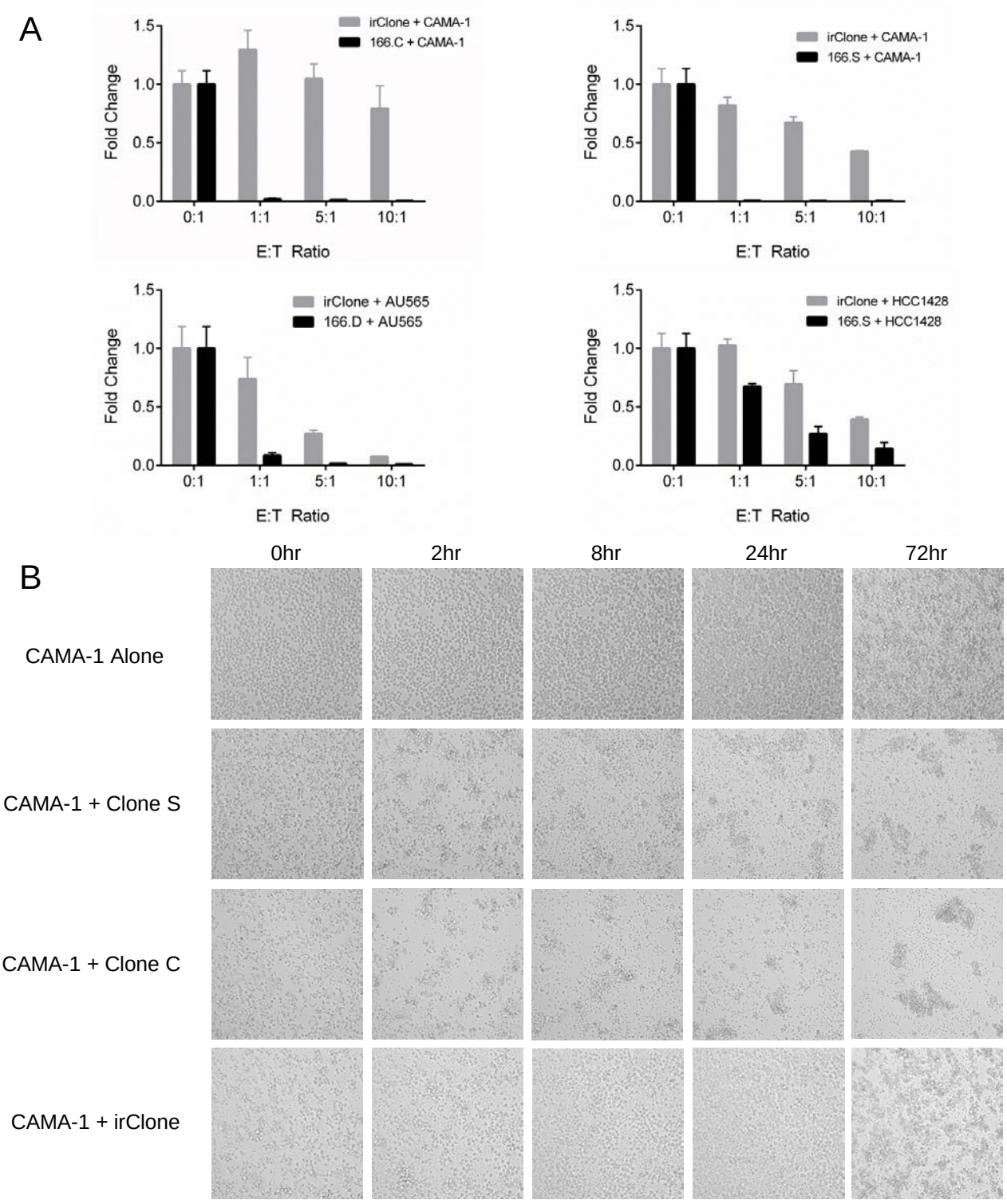


Figure 9. Patient BC166 clones kill breast cancer cell lines. T cell clones were assayed for their ability to kill breast cancer cell lines by flow cytometry counting of target cells after co-culture of cell lines and T cells at various ratios. Data was normalized to cell line only controls and displayed as fold change (A). Live cell imaging confirmed T cell mediated killing of cancer cell lines (B). Brightfield images shown are using a 10x objective.